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Ryukyu endemic *Mycalesis* butterflies, speciated vicariantly due to isolation of the islands since 1.55 Ma

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Abstract We analyzed the mitochondrial *COI* gene and drew phylogenetic trees of *Mycalesis* bushbrown butterflies in eastern Asia. In the Ryukyu islands, *Mycalesis madjicosa amamiana* is distributed on the Amami-Okinawa islands, and *Mycalesis madjicosa madjicosa* is distributed on the Yaeyama islands. In the phylogenetic tree, these Ryukyu endemic *M. madjicosa* species constitute a sister group with *Mycalesis gotama* distributed in Japan, Tsushima, southern Korea, southern China, Taiwan, and Viet Nam. This would reflect the isolation of the Ryukyu islands at 1.55 Ma, although speciation of *Mycalesis madjicosa* into two subspecies through the Kerama strait, and speciation of *Mycalesis gotama nanda* in Taiwan from the other *M. gotama* subspecies was probably delayed by 1 m.y. The Tsushima population of *M. gotama fulginia* is only slightly differentiated from the main Japan population, indicating that the Tsushima strait would act as only a mild barrier for *Mycalesis*.

Key words BEAST, China, Japan, MEGA, ML phylogenetic tree, raxmlGUI, Taiwan.

Introduction

Recent geologic investigations have shown that the Ryukyu islands were rapidly separated from the Chinese mainland as a result of the opening of the Okinawa trough beginning at 1.55 Ma (Osozawa *et al.*, 2012; Fig.1 A). Considering this newly proposed paleogeographic reconstruction, these geological investigators speculated that the isochronous isolation of the islands might have triggered vicariant speciation and produced the Ryukyu endemic species. If verified, this constitutes a major advance in understanding, for it would suggest that the high diversity of insects in the Ryukyu islands, the so-called Oriental Galapagos, may be a consequence of this island formation process that continues to the present.

Because each island has been isolated since separation from the mainland, each island ideally generates an endemic species from a common widely distributed ancestral continental species. We do not consider migration from the Asian continent by a persistent land bridge, because such a feature did not exist (Osozawa *et al.*, 2012). However, many insect species can fly, and gene flow is expected between two islands separated by narrower straits. In addition, at times of low sea levels due to glaciation, Okinawa-jima and Tokashiki-jima, and also Ishigaki-jima and Iriomote-jima, for example, may have been connected to form single islands.

The Kerama strait is a wide and deep strait also formed by rifting contemporaneous to that of the Okinawa trough (Osozawa *et al.*, 2012; Fig. 1A). It may have acted as a barrier even for these relatively strongly-flying species. The Tokara and Yonaguni straits are also such barriers (Fig. 1A). As a consequence, only two endemic species are expected in the Amami-Okinawa islands and the Yaeyama islands, respectively (Fig. 1A). In addition, the Tsushima and Taiwan straits, although presently shallow, have also existed since 1.55 Ma (Osozawa *et al.*, 2012; Fig. 1A), and an additional three endemic species are expected to have been generated in Japan, China-Korea, and Taiwan, respectively (Fig. 1A). The expected tree topology of the insects, based on this geological history, is shown in Fig. 1B.

If the above consideration is correct, the actual phylogenetic tree would furcate five ways at 1.55 Ma (Fig. 1B), and our hypothesis would be verified. If this is indeed the case, we can calibrate the fifth furcating node as 1.55 Ma, independent of the previously assumed base substitution rate (e.g., Papadopoulo *et al.*, 2010), and obtain a reliable evolution rate. Such a rate can be extrapolated to other species with uncertain calibration or with lesser geological information. Using deposited GenBank/DDBJ data, Osozawa *et al.* (2013) rebuilt and reevaluated phylogenetic trees based on the above-

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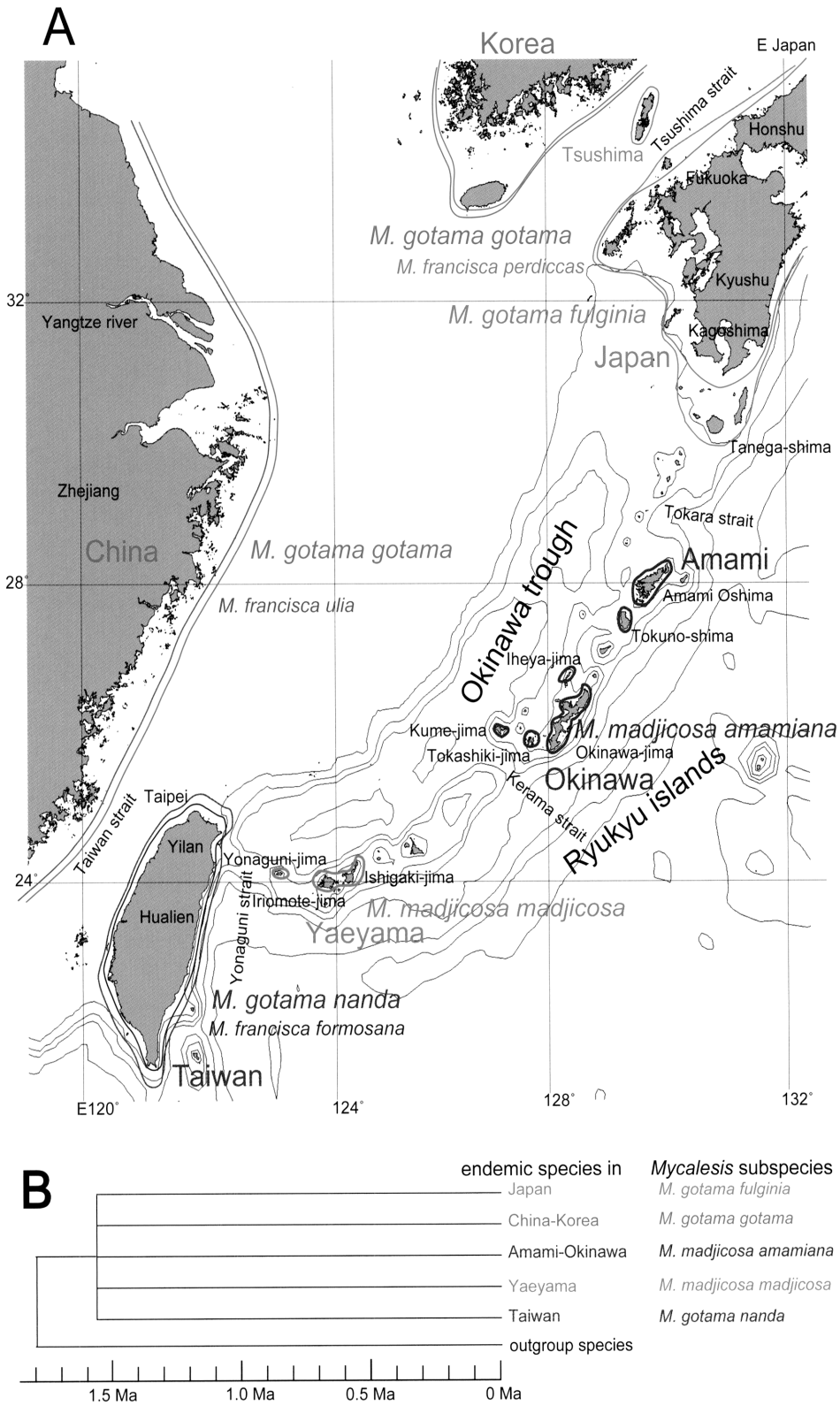

Fig. 1. A: Index map of Ryukyu islands and distributional map of *Mycalesis* species and subspecies. The islands were formed due to isolation from the Asian continent and subsidence, due to rifting and opening of the Okinawa trough starting at 1.55 Ma and continuing to the present. B: Expected tree topology of Ryukyu endemic insect species in Amami-Okinawa-and Yaeyama, but containing other endemic species in Japan, China-Korea, and Taiwan. Corresponding *Mycalesis* subspecies are shown. Fifth furcation at 1.55 Ma is a consequence of contemporaneous formation of sea straits barriers.

described geological model, and showed strong evidence for such vicariant speciation of four Ryukyu insect species. However, that tree basically furcates four ways. In that model, the Tsushima and Taiwan straits did not act as barriers for these species, whereas the Chinese central plain might have been a barrier.

In this paper we analyze the mitochondrial *COI* sequence of butterfly specimens of endemic *Mycalesis* species distributed in the Ryukyu islands, as well as those in Taiwan, Japan, Korea, China, and Viet Nam. It is valid to analyze species to species, because tree topologies have variations.

Mycalesis gotama Moore, 1858 (Lepidoptera, Nymphalidae, Satyrinae), analyzed in this paper, is an east Asian bushbrown butterfly species. It does not occur in northern China, and the populations in the Ryukyu islands were once considered a subspecies. By morphological, ecological analysis, and by experimentally crossing these species in Japan and Ryukyu, Takáhashi (1978) concluded that *Mycalesis gotama* Moore, 1958 is divided into two species; *Mycalesis madjicosa* Butler, 1868, endemic in Ryukyu, and *Mycalesis gotama* Moore, 1858 in the rest of eastern Asia. Takáhashi (1981) further divided the two species into several subspecies. *Mycalesis madjicosa* Butler, 1868 consists of *Mycalesis madjicosa amamiana* Fujioka, 1975 on the Amami and Okinawa islands, and *Mycalesis madjicosa madjicosa* Butler, 1868 on the Yaeyama islands (Fig. 1A) (See Takáhashi (1979) and a series of his papers for further details). *Mycalesis gotama* Moore, 1858 contains *Mycalesis gotama fulginia* Fruhstorfer, 1911 in Japan and Tsushima, *Mycalesis gotama gotama* Moore, 1858 in southern Korea (not collected and the data is from GenBank/DDBJ) and southern China, *Mycalesis gotama nanda* Fruhstorfer, 1908 in Taiwan, and *Mycalesis gotama charaka* Moore, 1874 in northern Indochina (simple distribution map is in Takáhashi, 1979). These subspecies are also analyzed in this paper. We call these bushbrown butterlies the *Mycalesis gotama* group.

We also analyzed another bushbrown butterfly, *Mycalesis francisca* (Stoll, 1780), although this species is not distributed in the Ryukyu islands. Its analyzed subspecies are; *Mycalesis francisca perdiccas* Hewitson, 1862 in Japan (not distributed in Tsushima, but in Okino-shima) and southern Korea (not amplified and the data is from GenBank/DDBJ), *Mycalesis francisca ulia* Fruhstorfer, 1908 in China, *Mycalesis francisca formosana* Fruhstorfer, 1908 in Taiwan. The other analyzed bushbrown butterfly species are; *Mycalesis zonata* Matsumura, 1909 and its grouped species, and *Orsotriaena medus medus* (Fabricius, 1775) is the outgroup species.

Materials and methods

Specimen numbers, species and subspecies names of *Mycalesis*, localities, collecting date, and collectors are registered in GenBank/DDBJ. Samples are mostly collected by the senior author. The accession numbers with specimen numbers, species and subspecies names, and simplified locality names are shown in Figs 2 and 3.

Whole body samples with wings were stored in ethanol collection bottles at normal temperature. However, in order to avoid degradation of DNA, we stored legs with some muscle in ethanol 1.5 mL tube, and the tube and resting bodies were stored in a freezer at –30°C.

Analytical methods are described in Osozawa and Oba (2013ab). DNA extraction was done using GenEluteTM Mammalian Genomic DNA Miniprep Kit by Sigma-Aldorich, and partly NucleoSpin® Tissue by Takara. *COI* primers are on the webpage of Barcoding of Animals, Japan Barcode of Life, Initiative (http://www.jboli.org/). LCO1490 GGTCAACAAATCATAAAGATATTGG, and HCO2198 TAAACTTCAGGGTGACCAAAAAATCA (Folmer *et al.*, 1994). According to the International Barcode of Life (http://www.ibol.org/), "the gene region that is being used for almost all animal groups, a 648 base-pair region in *COI*, is proving highly effective in identifying birds, butterflies (our present target), fish, flies and many other animal groups. The advantage of using *COI* is that it is short enough to be sequenced quickly and cheaply yet long enough to identify variations among species."

The above universal primers amplify a 648 bp *COI* sequence: its resolution is therefore sufficient and convenient for the purpose of barcoding animals including insects in order to investigate interspecies variations. The purpose of the present paper is to obtain an accountable phylogenetic tree with suitable base substitutions and to compare the topologies in each island population to the islands' isolation events at 1.55 Ma (Fig. 1B).

GoTaq Green Master Mix, Promega, but changed to G2 type, was used for PCR. Temperature of incubation was at 94°C for 60 seconds (twice for G2), denaturation at 94°C for 40 seconds, annealing at 45°C for 40 seconds, extension at 72°C for 60 seconds, cycled 5 times, in addition, denaturation at 94°C for 40 seconds, annealing at 51°C for 40 seconds, extension at 72°C for 60 seconds, cycled 35 times, and final extension at 72°C for 5 minutes, following Eppendorf PCR Program for *COI*

offered by the Consortium for the Barcode of Life. PCR product was purified using Wizard SV Gel and PCR Clean-Up System, Promega. Sequencing was done by Operon Biotechnology and Macrogen Japan, and more than 600 bp *COI* sequence was readable for each sample.

As noted in Osozawa *et al.* (2013), our evolutionary analyses were conducted using MEGA5 (Molecular Evolutionary Genetics Analysis 5; Tamura *et al.*, 2011). Base substitutions were checked to be not saturated by a function included in MEGA5. The Maximum likelihood (ML) tree was linearized to visualize the tree time increments. We confirm that the molecular clock test has been passed. Simultaneous branching time in the phylogenetic tree can be calibrated at 1.55 Ma corresponding to the time of isolation of the islands, and the evolution rate is strictly calculated without assumptions. It should be an attractive feature of the present paper, as well as Osozawa *et al.* (2013).

Topology check was done by drawing phylogenetic tree using raxmlGUI v1.3 (Randomized Axelerated Maximum Likelihood GUI; Silvestro and Michalak, 2011). To draw the tree, FigTree v1.4.2. was used.

The topology was also checked by BEAST v1.8.0 (Bayesian Evolutionary Analysis Sampling Trees; Drummond *et al.*, 2012). BEAUti v1.8.0, BEAST v1.8.0, Tracer v1.6, TreeAnnotator v1.8.0, and finally FigTree v1.4.2 were run. Substitution rate for *COI* calculated by MEGA5 was entered in BEAUti.

Results

Description

Phylogenetic trees for *Mycalesis* are shown on Figs 2 and 3. The ML tree made by MEGA5 is shown on Fig. 2A, and its linearized tree is shown on Fig. 3A. The ML tree made by raxmlGUI is shown on Fig. 2B, and the tree made by BEAST is shown on Fig. 3B. Tree topology is concordant between Fig. 2A and B, and between Fig. 3A and B, with the exception of a branch of *M. gotama gotama*, JX445984, southern Korea.

For the *Mycalesis* tree (Figs 2 and 3), *M. gotama* and *M. madjicosa* are differentiated into clade I (*M. gotama fulginia*, *M. gotama gotama*, *M. gotama charaka*; Japan, Tsushima, Korea, China, and Viet Nam), clade II (*M. gotama nanda*; Taiwan), clade III (*M. madjicosa amamiana*; Amai-Okinawa islands), and clade IV (*M. madjicosa madjicosa*; Yaeyama islands). Tree topology shows that these subspecies and the areal distribution is correspondent. Such correspondence is also found in the other *M. francisca* and *M. zonata* group.

Discussion

Vicariant speciation of *Mycalesis gotama* group

The tree topology made by MEGA5, raxmlGUI, and BEAST is concordant, and therefore reliable.

In the Ryukyus *Mycalesis*, *M. madjicosa amamiana* in Amai-Okinawa islands and *M. madjicosa madjicosa* in Yaeyama islands constitute two major clades III and IV (Figs 2 and 3). Contemporaneously, *M. gotama* divided into clade II of Taiwan and the other clade I of Japan, Tsushima, Korea, China, and Viet Nam (Figs 2 and 3).

We do not put 1.55 Ma at the nodes above, but at the node between the two major clades of *M. madjicosa* and *M. gotama* (Fig. 3A). The explanation is that only the barrier of the Okinawa trough and the Tokara and Yonaguni straits (Fig. 1A) were effective to cause vicariant speciation at 1.55 Ma, and that the Kerama and Taiwan straits (Fig. 1A) did not act as an effective barrier for *Mycalesis* at this time.

The habitat of *M. madjicosa* in the Ryukyu islands consists of dark forests, which are present on forested islands (Fig. 1A). In contrast, the habitat of *M. gotama* in Taiwan, Vietnam, China, and Japan is known to be in open rice fields on the side of hills (Takáhashi, 1979). The habitat difference between *M. madjicosa* and *M. gotama* is shown in tree topology (clades I and II vs clades III and VI; Figs 2 and 3).

Mycalesis gotama charaka in Viet Nam and especially *M. gotama gotama* in southern Korea is differentiated from the remaining *M. gotama* subspecies (Figs 2 and 3). *M. gotama gotama* in China is not differentiated from *M. gotama fulginia* in Japan (Figs 2 and 3). For the latter, the *COI* sequences are similar and stable among the Japanese populations, but the Tsushima population forms a minor geographical clade (Figs 2 and 3), indicating that the Tsushima strait may have existed as a very weak barrier. Consequently, the presently obtained fourth furcation (Figs 2 and 3) does not accord with the expected fifth furcation (Fig. 1B).

Topology of *Mycalesis francisca* and *zonata* group

Mycalesis francisca is a forest dwelling species (Fukuda *et al.*, 1992) with a habitat similar to *M. madjicosa*, but contrasting with *M. gotama*. *M. francisca perdiccas* in Japan and South Korea and *M. francisca ulia* in China have similar *COI* sequences like as *M. gotama*, but *M. francisca formosana* in Taiwan is distinct and constitutes a different clade (Figs 2 and 3). Larvae from Taiwan are morphologically different from Japanese larva (Takáhashi, 1987). Apparently in northern and eastern

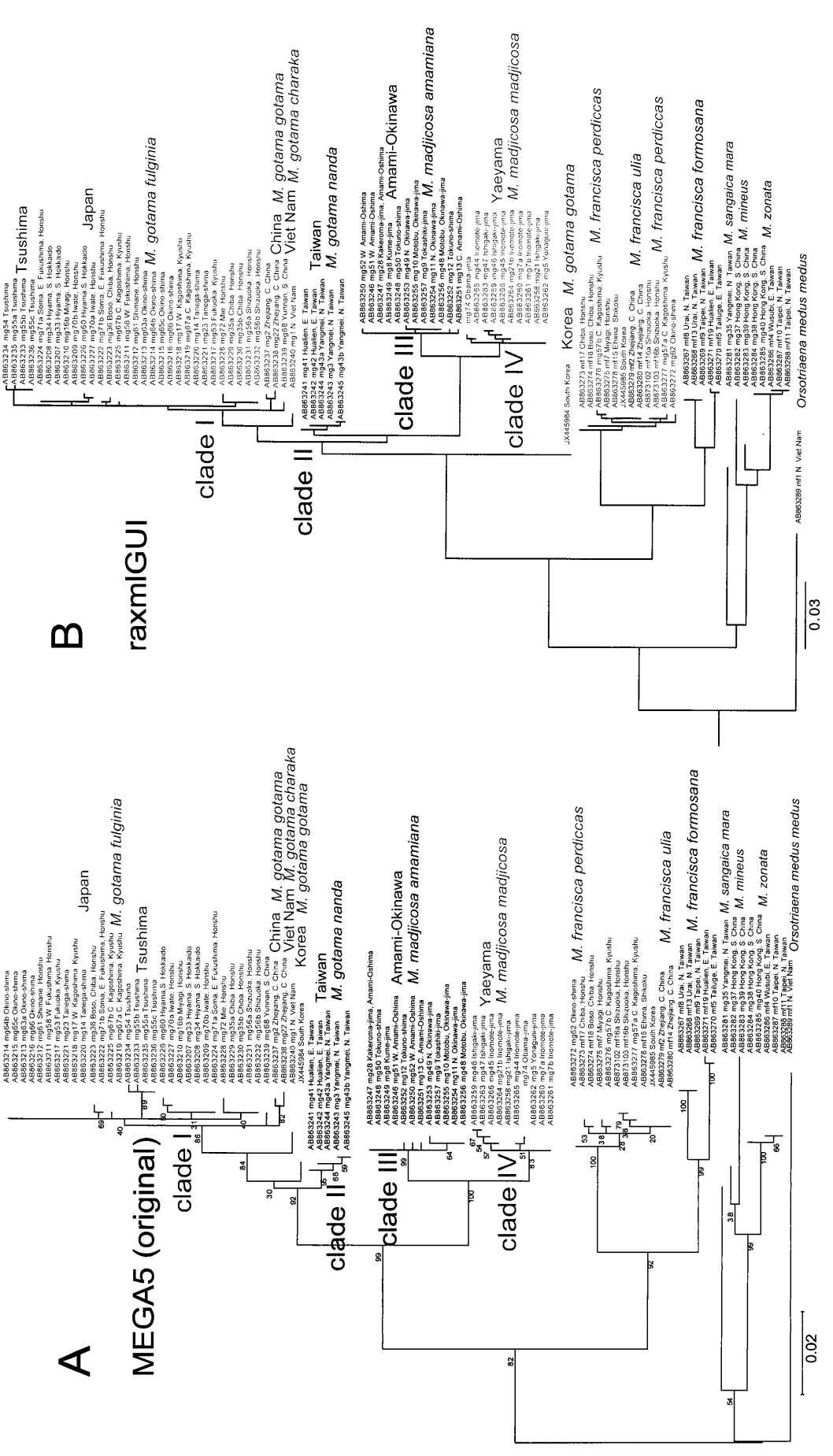

Fig. 2. A: The phylogenetic ML tree of *Mycalesis* species and subspecies using mitochondrial *COI* sequence (624 bp), constructed using MEGA5. B: ML tree constructed using raxmlGUI.

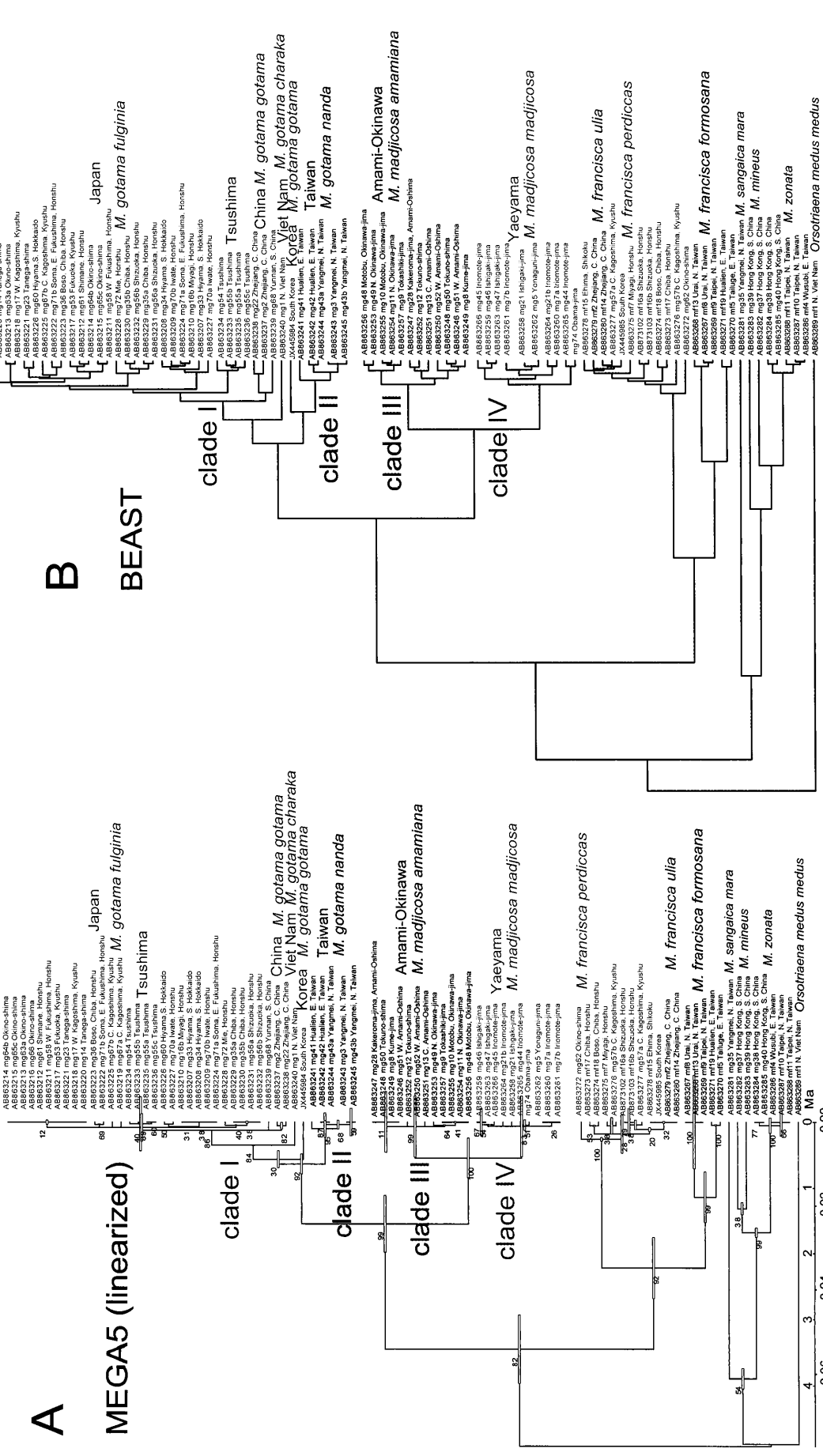

Fig. 3. A: The linearized phylogenetic ML tree of *Mycalesis* species and subspecies using *COI* gene sequence (624 bp). Highest Log likelihood= − 2784.31. The molecular clock is not rejected with $p < 0.8151$. Bootstrap values are shown at each branch. The 95% confidence interval of nodes is shown as rectangles. Evolution rate is 1.56 % /m.y.
B: The phylogenetic tree was constructed using BEAST.

Taiwan, *M. francisca formosana* is deeply differentiated into two populations (Figs 2 and 3), although these two are externally indistinguishable. The Yilan basin is a western extension of the Okinawa trough (Fig. 1A), and may have acted as a barrier to differentiate *M. francisca formosana*.

Mycalesis zonata and its group build a major distinct clade from the other *Mycalesis* species, but *M. zonata* itself has similar sequences between Taiwan and China (Figs 2 and 3).

Evolutionary rate

According to Fig. 3A, base substitution rates of *COI* gene are estimated to be 1.56 %/m.y. for *Mycalesis*. These values are comparable to those estimated and compiled by Osozawa *et al.* (2013), and 1.77 %/m.y. was assumed by Papadopoulo *et al.* (2010).

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摘　要

琉球固有種としての*Mycalesis*属のチョウ，それらの1.55Ma島嶼化による異所的種分化（遅沢壮一・高橋真弓・John WAKABAYASHI）

琉球の島嶼化は沖縄トラフとそれに付随する海峡群の形成によるが，対馬海峡と台湾海峡も含めて，1.55Maに開始していると考えられる．琉球を含む*Mycalesis*属についての線形化したML樹形図では，*Mycalesis gotama* のグループについては，1.55Maの同時5分岐が期待できる．これらのミトコンドリア*COI*領域の配列を得て，MEGA5以外にも，raxmlGUIとBEASTによる樹形図を作成した．対馬海峡は障壁として不十分で，結果は4分岐であったが，異所的種分化を支持するものである．またその*COI*についての塩基置換速度も正確に求まった．上記グループ以外の他の*Mycalesis*の種についても樹形図を作成し，系統を調べた．

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